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Altered Viral Ribonucleic Acid Synthesis by a Temperature-sensitive Mutant of Eastern Equine Encephalitis Virus

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A temperature-sensitive mutant (*Ets-4*) derived from eastern equine encephalitis virus induced the synthesis of unusually large amounts of viral RNA in infected chick embryo cells. *Ets-4* produced two to four times more RNA at 37°C; however, its yield of mature virus was about 10 times less than the parent virus. Viral RNA extracted from infected chick embryo cells was fractionated on sucrose gradients. Three types of viral RNA were synthesized by both viruses: the infectious 45 s RNA, 27 s "interjacent" RNA and a 20 s ribonuclease resistant double-stranded RNA.

Ets-4 produced greater amounts of each RNA species than did encephalitis virus. Production of the 45 s infectious RNA by *Ets-4* as measured by plaque assay was less than that of the parent virus, but the incorporation of radioactive uridine into this RNA species was greater, suggesting that the 45 s RNA was biologically defective. On a specific infectivity basis (infectious 45 s RNA/radioactive 45 s RNA) the 45 s RNA of *Ets-4* possessed only 2% of the infectivity observed for its parent. Viral protein synthesis, measured by the complement fixation and hemagglutinin tests, induced by *Ets-4* was depressed below that of the parent virus indicating that the increased RNA synthesis of the mutant did not result in correspondingly increased amounts of virus protein. The apparent defect in viral protein synthesis may be the result of a defect in viral RNA made by *Ets-4*.

On the basis of the information available, it appeared that viral RNA synthesis in *Ets-4* infected cells was out of control. Control seemed to be restored to the level and pattern of the parent by superinfection with the parent encephalitis virus or Venezuelan equine encephalitis virus but not with other viruses.

1. Introduction

A number of reports have appeared which indicate that after infection of cells by group A arboviruses, a number of different classes or species of viral RNA are formed (Martin, 1966; Sreevalsan & Lockart, 1966; Friedman, Levy & Carter, 1966; Sonnabend, Martin & Mecs, 1967; Burge & Pfefferkorn, 1967; Dobos & Faulkner, 1969). Generally the classes or species of RNA have been divided on the basis of their sedimentation coefficients calculated from data obtained after sucrose-gradient centrifugation. Thus far, available data indicate that double-stranded or multi-stranded forms (16 to 20 s)‡ of viral RNA are formed initially in the infected cell and that almost

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‡ No distinction is made in this paper between the replicative form (RF) and the replicative intermediate (RI) (Franklin, 1967). The former is double-stranded RNA and the latter may be a double-stranded RNA with nascent single strands attached. In this paper, both forms will be described together as 20 s, since they were observed as one peak after centrifugation in a sucrose gradient.

simultaneously or soon thereafter a single-stranded relatively non-infectious "interjacent"† 27 s RNA is found. This is followed by the appearance of a single-stranded infectious 45 s species associated with the mature virus particle. There are a few variations on this theme, and the precise nature, function and interrelationship of each species are not completely known. Furthermore, little information is available on control of viral RNA synthesis. The present paper is a report on an abnormal pattern of viral RNA synthesis induced in chick embryo cells by a temperature-sensitive mutant (*Ets-4*) of eastern equine encephalitis virus at 37°C. Compared with its parent, the mutant appears to have lost control of the synthesis of its RNA. Control can apparently be restored to equal that of the parent by superinfection with the parent or Venezuelan equine encephalitis virus, but not by certain other viruses.

2. Materials and Methods

(a) *Cell cultures, virus infection and growth*

These procedures have been described previously in detail for certain group A arboviruses and chick embryo cells (Brown, 1963; Zebovitz & Brown, 1967).

(b) *Virus strains*

The parent virus was the Louisiana strain of eastern equine encephalitis virus whose properties have been described by Brown (1963) and by Zebovitz & Brown (1967). Chick embryo cell culture seeds usually contained between 1 and 8×10^9 plaque forming units per ml.

The temperature-sensitive mutant, *Ets-4*, was obtained after treating with nitrous acid the infectious RNA that was isolated from the parent virus (Burge & Pfefferkorn, 1966). The nitrous acid treatment resulted in a 99% reduction in infectious RNA titer. Chick embryo cell monolayers were infected with the treated infectious RNA, overlayed with a lactalbumin hydrolysate agar medium (Zebovitz, 1965), and incubated at 30°C Plaques which appeared were picked at random and replaqued in duplicate sets of plates, one of which was incubated at 30°C and the other at 42°C. Those isolates which formed plaques at 30°C but not at 42°C were further purified three times by replaquing; seeds were then made in chick embryo cells at 30°C.

Such seeds usually contained between 1×10^9 to 1×10^{10} plaque forming units/ml. and those that had a reversion frequency (ratio of number of plaques formed at 42°C to number of plaques formed at 30°C) of less than 10^{-4} were selected as candidates for temperature-sensitive mutants. Only after it was demonstrated that multiplication of the candidate virus was inhibited significantly at 42°C in liquid monolayer cultures were the candidates accepted as valid mutants, since occasionally clones were encountered that could not plaque at 42°C under agar but that were only slightly inhibited in growth at this temperature in liquid monolayer cultures. *Ets-4* was one of the mutants selected.

In certain experiments, *Ets-4*-infected cultures were superinfected with a number of different viruses. These included the following viruses from our laboratory chick embryo cell-culture stocks: Venezeulan equine encephalitis (Trinidad), Sindbis (AR339), Semliki Forest, vesicular stomatitis virus (New Jersey), and Newcastle disease virus.

(i) *Methods of viral RNA analysis*

Viral RNA synthesis in the presence of actinomycin D was measured by the incorporation of radioactive uridine into an acid-precipitable product extracted from infected chick embryo cells by hot phenol and sodium dodecyl sulfate. Chick embryo cells in

† The interjacent RNA (Martin, 1966) has been calculated to have a value of 27 s. and the infectious RNA, 45 s. In the papers of other arbovirus workers, an interjacent RNA has been described as 26 s to 28 s, and the highly infectious RNA as 40 s to 47 s.

60 mm Petri plates were infected at a multiplicity of infection of 10 plaque forming units/cell, and overlayered with 2 ml. of a serum-free minimal medium (Hanks balanced salts solution; cystine, 75 mg/l.; histidine, 60 mg/l.; sodium bicarbonate, 0·14% (Zebovitz, 1965) containing 0·1 μg actinomycin D/ml. and [^{14}C]uridine (0·025 μc/ml.). Insulin at 0·27 unit/ml. was added to the medium to enhance the incorporation of radioactive uridine (Cooper, 1966). Cultures were incubated at 37°C in an incubator supplied with a 5% CO_2 and 95% air mixture. At 2-hr intervals 5 plates were removed from the incubator and supernatant medium removed. The cells were washed twice with phosphate-buffered saline pH 7·4 and viral RNA was extracted in the manner described below. The RNA was dissolved in PO_4-buffered saline; 100 μg yeast RNA was added as carrier and precipitated with 10 ml. of 10% trichloroacetic acid. This precipitate was trapped and washed on Millipore filters which were then dried in an oven. Radioactivity was subsequently determined with a Packard liquid-scintillation spectrometer.

(ii) *Extraction of viral RNA*

Virus-infected cells were overlayed with 2 ml. of the serum-free minimal medium described above containing either [^{14}C]uridine (0·025 μc/ml.) or [5-^{3}H]uridine (1 μc/ml.). The cultures were incubated at 37°C for 6 hr. The cells were scraped off the plates and suspended in 0·02 M-phosphate and 10^{-3} M-EDTA, pH 7·4. Viral RNA was extracted from the cells with hot (60°C) water-saturated phenol containing 0·5% sodium dodecyl sulfate (Girard, 1967). Two phenol extractions were performed and the aqueous phase was precipitated with 3 vol. ethyl alcohol containing 2% potassium acetate. The RNA was stored in alcohol at −70°C until required. The RNA precipitate was removed from the alcohol by centrifugation and dissolved in 2 ml. of PO_4-buffered saline.

(iii) *Assay of infectious RNA*

Viral RNA extracted as described above was assayed on chick embryo cell monolayers made hypertonic by washing once each with PO_4-buffered saline, 0·5 M-NaCl, and then with 1·0 M-NaCl–0·1 M-Tris–HCl buffer, pH 8·2 (Colon & Idoine, 1964). RNA dilutions were made in the Tris-buffered 1·0 M-NaCl. A sample (0·1 ml.) of the diluted RNA was put on the hypertonic monolayers and allowed to fix for 15 min; the inoculum was removed and the monolayers overlayered with lactalbumin hydrolysate agar. Cultures were incubated for 2 days at 37°C, stained with neutral red, and plaques scored a few hours later.

(iv) *Sucrose density-gradient analysis*

Two-ml. samples of viral RNA in PO_4-buffered saline were layered on a 28-ml. sucrose gradient (15 to 35%) made in reticulocyte standard buffer (0·01 M-Tris–HCl, pH 7·4; 0·01 M-KCl; 0·0015 M-$MgCl_2$), (Warner, Knopf & Rich, 1963), containing 2 μg polyvinylsulfate/ml. and 0·15 M-NaCl. The gradients were then centrifuged at 22,000 rev./min in an SW25 rotor of the model L2 Spinco ultra-centrifuge for 18 hr. Ten-drop fractions were collected from the bottom of the tube. Each fraction was analyzed for optical density at 260 nm to determine the location of the 28 and 18 s ribosomal RNA peaks. Radioactive measurements were made as described above after precipitating the viral RNA in each fraction with 10% trichloroacetic acid. Ribonuclease-free sucrose was obtained from Mann Research Laboratories.

When it was desired to determine the infectious RNA content of the fractions, 0·1 ml. of each fraction was removed immediately after collection and the titer was determined in the manner described earlier for assay of infectious RNA.

RNase resistance of viral RNA in the sucrose gradient fractions was determined in the following manner. The liquid volume of each fraction was adjusted to 2 ml. with sterile distilled water, and divided into two tubes of 1 ml. each. One set of tubes was processed directly for radioactivity by the addition of 10% trichloroacetic acid as described above. The second set of tubes was treated with 2μg of pancreatic RNase added in 1 ml. distilled water to give a final concentration of 1 μg RNase/ml., NaCl at approximately 0·025 M and 0·005 M-Mg^{2+}. The samples were incubated in a 37°C water bath for 30 min and processed in the manner described above for radioactivity. Separate experiments in which the digestion was carried out in the presence of 0·15 M-NaCl gave similar results.

(v) *Tests for complement-fixing antigen and hemagglutinin*

Complement-fixing antigen present in infected cells was released by scraping the cells off the plates into PO_4-buffered saline. The cells were pooled in a test tube, and disrupted for 30 sec with a Branson sonic oscillator. The cell debris was removed by centrifugation and a complement-fixing test was performed on the cell extract according to the standardized diagnostic method of the U.S. Public Health Service monograph no. 74 (1965). The antiserum was obtained from guinea pigs immunized with infectious encephalitis virus that had been purified by centrifugation in a sucrose gradient. This antiserum did not react with host-cell components and specifically detected the presence of virus antigen in infected cell extracts. It is not known how many of the virus-induced proteins the complement-fixing test measures. The hemagglutinin test (Clarke & Casals, 1958) was performed with serum-free tissue culture fluids (minimal medium, Zebovitz, 1965) derived from infected chick embryo cell cultures.

3. Results

(a) *Growth response and RNA synthesis of encephalitis and* Ets-4 *viruses*

The growth curves and the incorporation of [^{14}C]uridine by encephalitis virus and its temperature-sensitive mutant are compared in Figure 1. Samples of the overlay medium were taken at two-hour intervals and assayed for virus titers on chick embryo cells. Encephalitis virus yields high titers on chick embryos cells, between 9·0 and

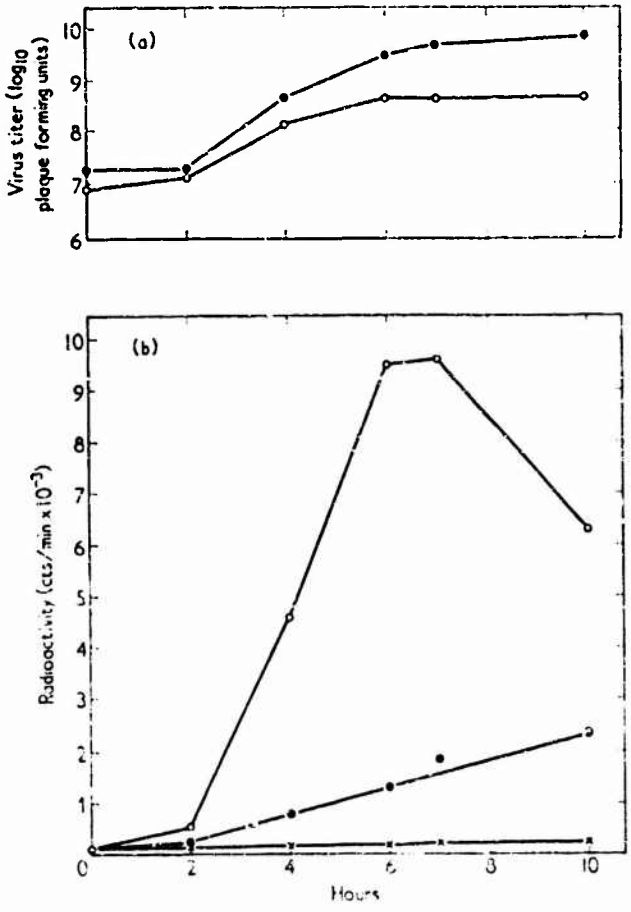

FIG. 1. (a) Growth response and (b) RNA synthesis at 37°C in chick embryo cells infected with parent and *Ets-4* viruses. --●—●—, Parental strain; —○—○—, *Ets-4*; —×—×—, uninfected chick embryo cells.

10·0 $\log_{10}$ plaque forming units within 10 hours at 37°C (Fig. 1(a)). *Ets-4* on the other hand consistently produces approximately one $\log_{10}$ plaque forming unit less virus. The replication of the mutant was more strongly influenced by temperature. *Ets-4* multiplication was almost completely inhibited at 42°C, yet the parent virus at this temperature attained titers almost equal to those at 37°C.

In the light of the results above, it was next of interest to compare the viral RNA synthesis of the mutant with that of the parent at 37°C. Figure 1(b) shows the incorporation of [^{14}C]uridine into an acid-insoluble RNA extracted from chick embryo cells infected with encephalitis and *Ets-4* viruses. The results indicate that *Ets-4*, which induced lower infectious titers of mature virus was able to synthesize considerably more viral RNA than its parent. These results indicated that *Ets-4* probably possesses a defect in its viral RNA synthesis and/or in its control mechanism.

To prove that the synthesis of RNA observed here was induced by the virus, a control of uninfected chick embryo cells was included in the experiment and treated in the manner described above for infected cells. In the experiment illustrated in Figure 1(b), the incorporation of uridine into cellular RNA never exceeded 200 cts/min. The incorporation of uridine observed in infected cells therefore is the result of the virus activity in the host cell and most likely represents virus specific RNA synthesis.

(b) *Viral protein synthesis*

To examine *Ets-4* virus in more detail, a series of experiments were performed to compare viral protein and RNA synthesis to that of the parent. In the first group of experiments the production of virus-specific complement-fixing antigen and hemagglutinin were used as an indication of protein synthesis. Table 1 shows the

TABLE 1

Complement-fixing antigen and hemagglutinin formation by parent strain and mutant Ets-4 *virus at 37°C*

Time (hr)	Complement-fixing antigen†		Hemagglutinin‡	
	E	*Ets-4*	E	*Ets-4*
0	0	0	0	0
2	0	0	0	0
4	4	2	64	32
6	32	8	512	64
10	64	32	1024	128
24	32	8	4096	128

† E, eastern equine encephalitis virus.
‡ Reciprocal of dilution.

results of a typical experiment. *Ets-4* produced less complement-fixing and hemag glutinin than was observed for the parent strain.

These data show that *Ets-4* viral protein synthesis did not keep pace with the increased amount of RNA it was able to make and was consistently less than the protein made by the parent strain.

(c) *RNA sedimentation patterns in sucrose gradients*

In an effort to analyze further the nature of the viral RNA formed by encephalitis virus and its mutant, *Ets-4*, an experiment was performed in which chick embryo

cells infected with either of the two strains were incubated at 37°C in the presence of [^{14}C]uridine and actinomycin D for six hours. The RNA was extracted from the infected cells, dissolved in PO_4-buffered saline, and fractionated by sucrose-gradient centrifugation. The sedimentation patterns of RNA from both viruses are shown in Figure 2. The position of ribosomal RNA peaks from chick embryo cells was determined by optical density measurements. Ribosomal RNA peaks of 28 s and 18 s

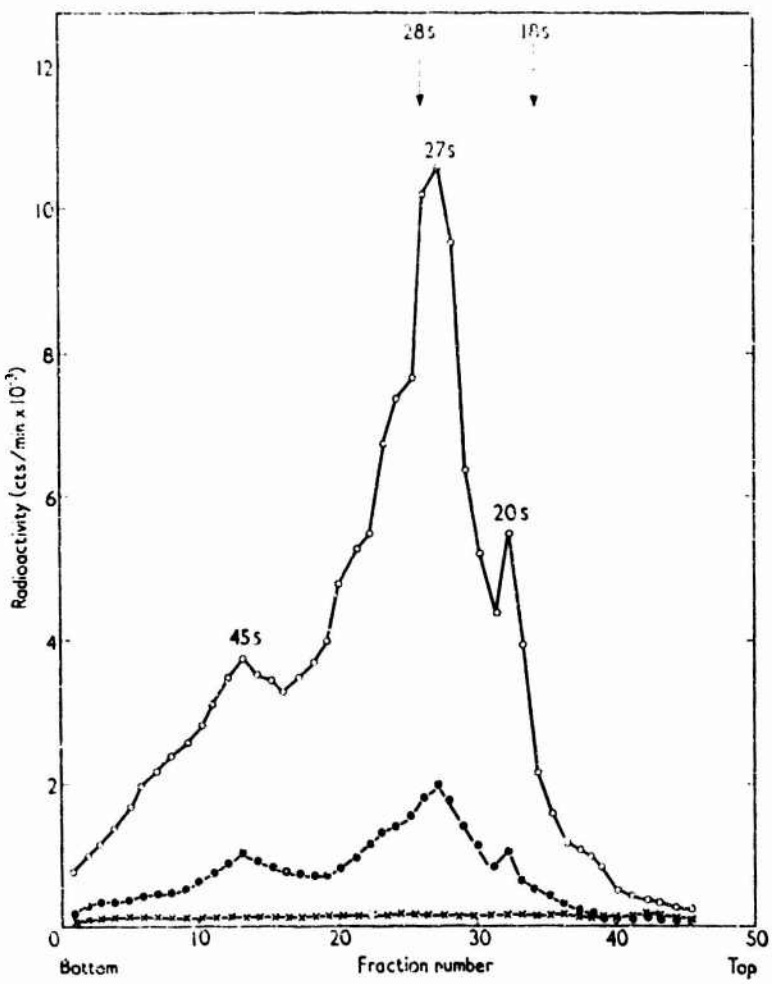

FIG. 2. Sucrose-gradient sedimentation of viral RNA extracted from parent- and *Ets-4*-infected chick embryo cells grown at 37°C for 6 hr. —●—●—, Parental strain; —○—○—, *Ets-4*; —×—×—, uninfected chick embryo cells.

species are indicated by arrows on this curve and were used as reference points to calculate the sedimentation coefficients (Martin & Ames, 1961) of the radioactive viral RNA peaks. Three major viral RNA peaks with sedimentation coefficients of 45, 27 and 20 s, respectively, were observed for both viruses. The amount of each RNA species made by *Ets-4* was consistently from two to four times greater than that made by the parent virus. The properties of each RNA species will be discussed later. The sedimentation pattern of RNA extracted from normal uninfected chick embryo

cells treated with actinomycin D in the same manner as the virus infected cells is also shown in Figure 2. A very low level of radioactive RNA was obtained from these cells and no sedimentation pattern was observed.

(d) *Relative infectivity of 45 S RNA*

The peak that is characterized by a value of 45 s is the infectious RNA that is incorporated into the mature virus particle (Dobos & Faulkner, 1969). About 98% of the infectivity found in viral RNA resides in this RNA species. Note that the total amount of *Ets-4* 45 s RNA as measured by radioactivity was much greater than that of the parent strain (Fig. 2), yet the infectivity of this RNA was found to be lower for *Ets-4*. A series of experiments were performed in which the relative infectivity of the 45 s RNA of both viruses was compared. Initial experiments were performed on 45 s RNA after fractionation of viral RNA obtained from infected cells on sucrose gradients. A specific infectivity value was calculated for each virus by the formula shown in the legend of Table 2. The value for 45 s RNA obtained from *Ets-4* by this

TABLE 2

Specific infectivity of 45 S RNA extracted from purified suspension of parent and Ets-4 *viruses*

Virus	Infectious RNA titer (plaque forming units/ml.)	Radioactivity of infectious RNA (cts/min/ml.)	Specific infectivity† (plaque forming units/cts/min)	Specific infectivity ratio‡
Parent strain	$6{\cdot}2\times10^6$	343,000	18	
Ets-4	$7{\cdot}5\times10^3$	24,900	0·3	0·02

† Specific infectivity $= \dfrac{\text{Infectious RNA titer}}{\text{Infectious RNA radioactivity}}$

‡ Specific infectivity ratio $= \dfrac{\text{Specific infectivity mutant}}{\text{Specific infectivity parent}}$

method had only 10 to 30% of the specific infectivity calculated for the parent. The values obtained in these experiments were at best an estimate of the relative infectivity of the two viruses, because part of the radioactivity observed in the 45 s peak fractions derived from sucrose gradients was contributed to by the adjacent 27 s RNA.

These experiments were modified so as to isolate 45 s RNA free from other forms of viral RNA by extracting it from purified virus suspensions made radioactive with [5-^{3}H]uridine. In this way a more precise comparison of the relative infectivity of the infectious RNA of the two viruses could be made. Virus was obtained from 18 hours growth on chick embryo cells overlayered with minimal medium containing [5-^{3}H]uridine (5 μc/ml.) and actinomycin D. The virus was concentrated by centrifugation and purified through sucrose gradients. RNA was extracted from these preparations as described in Materials and Methods with hot phenol and sodium dodecyl sulfate. Radioactivity measurements and infectious RNA titers were performed on 0·1-ml. samples in triplicate. The results of this experiment are shown in Table 2. The infectious titer and radioactivity associated with the parent virus was substantially greater than that of *Ets-4* but this is a reflection of the better growth capacity

of the parent. To compare the results of both viruses, a specific infectivity value was calculated relating infectivity to radioactivity. The specific infectivity of RNA from encephalitis virus was 18 plaque forming units/cts/min. That of *Ets-4* on the other hand was 0·3 plaque forming unit/cts/min. Thus there was less infectivity associated with *Ets-4* RNA than that of the parent strain. When the ratio of the two specific infectivity values was calculated by the formula shown in the legend of Figure 2 it was found that the RNA extracted from *Ets-4* had only 2% of the specific infectivity of RNA from the parent strain. These results confirm that the RNA made by *Ets-4* was biologically defective with respect to infectivity.

(e) *Characterization of viral RNA species*

Figure 3 demonstrates how the identity of each viral RNA peak was established. RNA extracted from chick embryo cells infected for six hours with *Ets-4* in the presence of actinomycin D and [5-^{3}H]uridine was fractionated on a sucrose gradient.

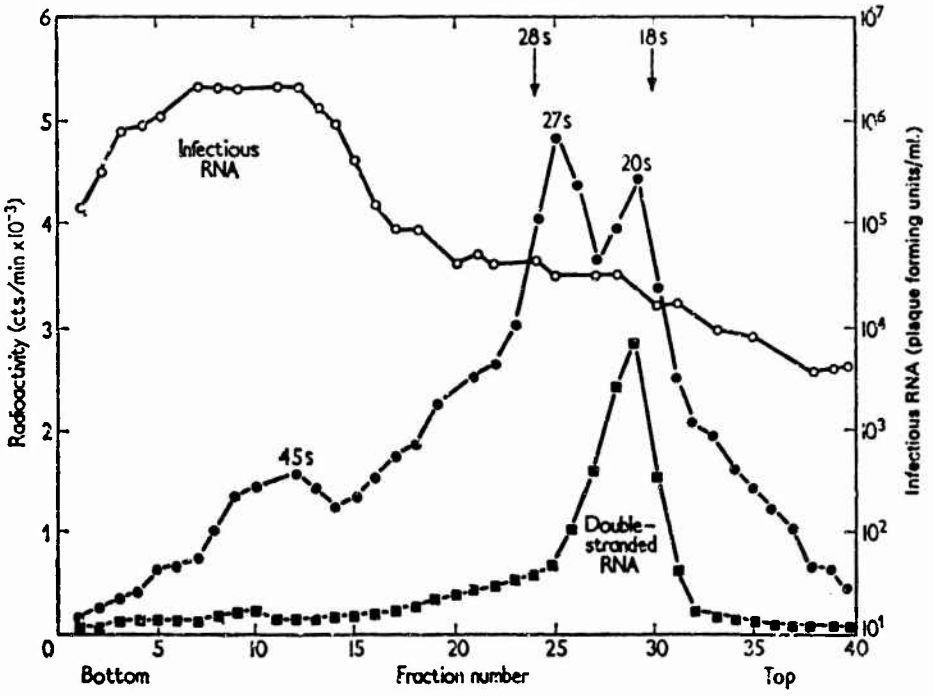

FIG. 3. Characterization of viral RNA peaks observed in sucrose gradients. *Ets-4*-infected chick embryo cells overlayered with minimal medium containing [5-^{3}H]uridine (1 μc/ml.) and actinomycin D (1 μg/ml.) were incubated at 37°C for 6 hr. Viral RNA was extracted, fractionated on sucrose gradients and assayed for viral RNA, infectious RNA, and RNase resistance as described in the Materials and Methods section. —●—●—, Viral RNA (cts/min); —○—○—, infectious RNA (plaque forming units/ml.); —■--■—, RNase-resistant RNA (cts/min).

Ten-drop fractions collected by bottom puncture of the gradient tube were each analyzed for radioactivity, infectious RNA, and RNase resistance. The peak infectivity was associated with the 45 s peak. In a separate experiment with the parent virus it was found that 1% of the total infectivity was found in the 27 s peak and that the 20 s peak appeared to be completely non-infectious. This is consistent with the work of others (Sonnabend *et al.*, 1967; Sreevalsan & Lockart, 1966).

The position of the double-stranded RNA was determined by treating each fraction with 1 μg RNase/ml. The RNase-resistant RNA was identical with the 20 s RNA peak indicating that the 20 s peak is probably composed primarily of double-stranded RNA. It is believed at this time that the 20 s RNA is a mixture of the replicative form and replicative intermediate.

It is possible to identify the 45 s RNA by its infectivity and the 20 s RNA by its RNase resistance. The 27 s RNA is more difficult to characterize because there are no known biological properties by which it can be measured. This RNA had little infectivity associated with it, and may be a transitional form of RNA that leads to the fully infectious 45 s RNA (Sreevalsan, Lockart, Dodson & Hartman, 1968). Dobos & Faulkner (1969) report that the 26 s RNA derived from Sindbis virus-infected BHK-21-C13 cells appears to act in polyacrylamide gel electrophoresis as a heterogeneous population of RNA molecules of different sizes and shapes. The 27 s RNA found here is probably single-stranded because it is easily destroyed by RNase.

It appears that *Ets-4* induced the synthesis of the same types of viral RNA as did the parent virus, but in much larger amounts, which, to emphasize again, did not result in correspondingly greater amounts of either infectious RNA or infectious virus particles.

(f) *Control of* Ets-4 *RNA synthesis by superinfection with the parent strain*

As a working hypothesis, one may consider the unusual amount of *Ets-4* RNA synthesis as indicating a loss of control by the mutant of its own RNA synthesis that results in the formation of large amounts of poorly infectious RNA. The parent virus presumably possesses a control mechanism that limits the production of viral RNA to normal levels. One could anticipate, therefore, that it might be possible to restore control of RNA synthesis to normal levels, by superinfection of *Ets-4* infected cells with the parent virus. The latter may provide the necessary control mechanism to limit *Ets-4* RNA synthesis to normal levels. Presumably, the mechanism is absent or defective in *Ets-4* infected cells. The results of an experiment to test these ideas are shown in Figure 4.

This Figure shows the sedimentation pattern of RNA extracted from chick embryo cells infected at the same time and with approximately equal multiplicity of parent and *Ets-4* viruses. The input multiplicity of infection in this experiment was adjusted to 10 plaque forming units/cell. The viruses were allowed to replicate together for six hours. The RNA sedimentation pattern of the doubly-infected cells remained the same in that the same peaks were still observed, but the amount of viral RNA formed in these cells was reduced to a level that closely approximated that of the parent strain alone. These data support but do not prove the notion that control was restored. The question arises whether or not the restoration of control is only apparent and whether, instead, the pattern of RNA synthesis in doubly infected cells was due to interference of *Ets-4* growth by parent virus and not directly related to the fundamental regulatory mechanism of viral RNA synthesis. This alternative explanation is considered unlikely because direct tests for interference (Zebovitz & Brown, 1968) in this experiment, and later ones with other group A arboviruses, revealed no difference in the growth curves of *Ets-4* or its parent in singly- or in doubly-infected cells; in fact, it was somewhat disappointing to find that the apparent restoration of control did not result in an increase in infectious *Ets-4*. Furthermore, in an extensive study of interference among group A arboviruses in cells treated with actinomycin D

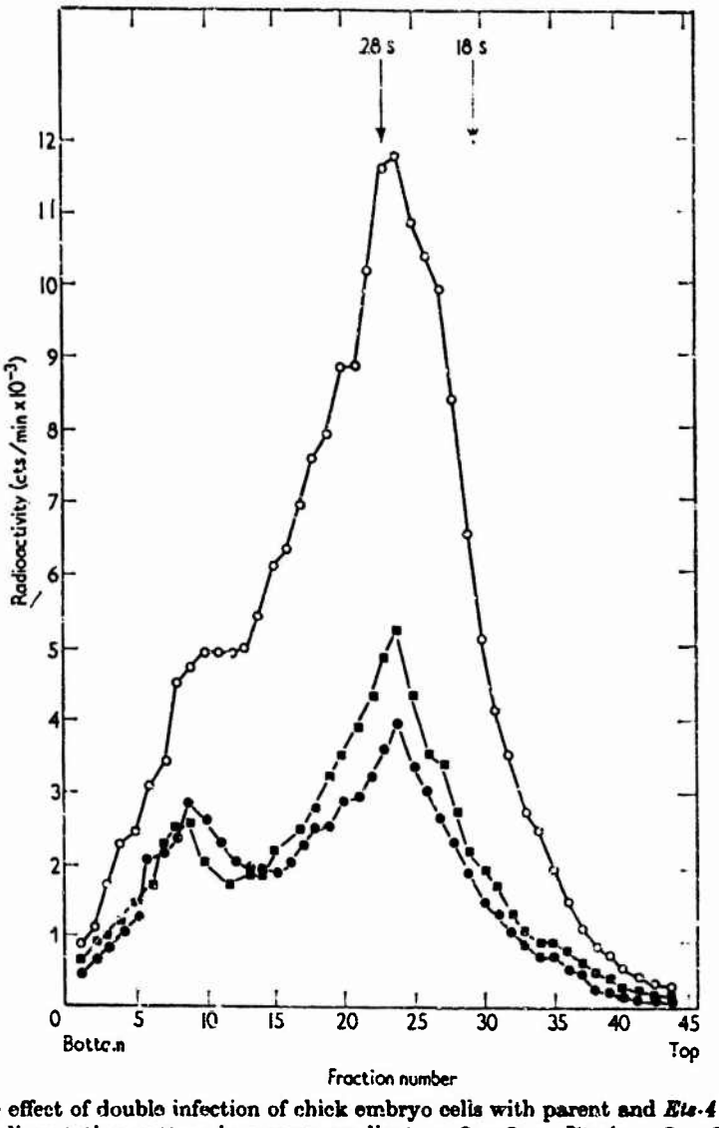

FIG. 4. The effect of double infection of chick embryo cells with parent and *Ets-4* viruses upon viral RNA sedimentation pattern in sucrose gradient. —○—○—, *Ets-4*; —●—●—, parental strain; —■—■—, *Ets-4* plus parental strain.

(Zebovitz & Brown, 1968) interference occurred only if one virus had an advantage over the other in time or in multiplicity of infection. Encephalitis virus and its mutant, *Ets-4*, were no exception in this regard; thus interference was apparently not involved in the experiments reported above.

(g) *Effect of time upon control of* Ets-4 *RNA synthesis by the parent virus*

The next experiment was designed to determine the time limit during which superinfection with the parent strain could dominate the pattern of viral RNA synthesis in cells doubly infected with parent and *Ets-4*. The data in Figure 5 show that superinfection was effective if parent virus was added up to one hour but not two hours after *Ets-4*. It is possible that at two hours post-infection the apparent failure to exert control of *Ets-4* RNA synthesis by the parent virus might be due to other factors such as interference by *Ets-4* of parental virus growth that may be

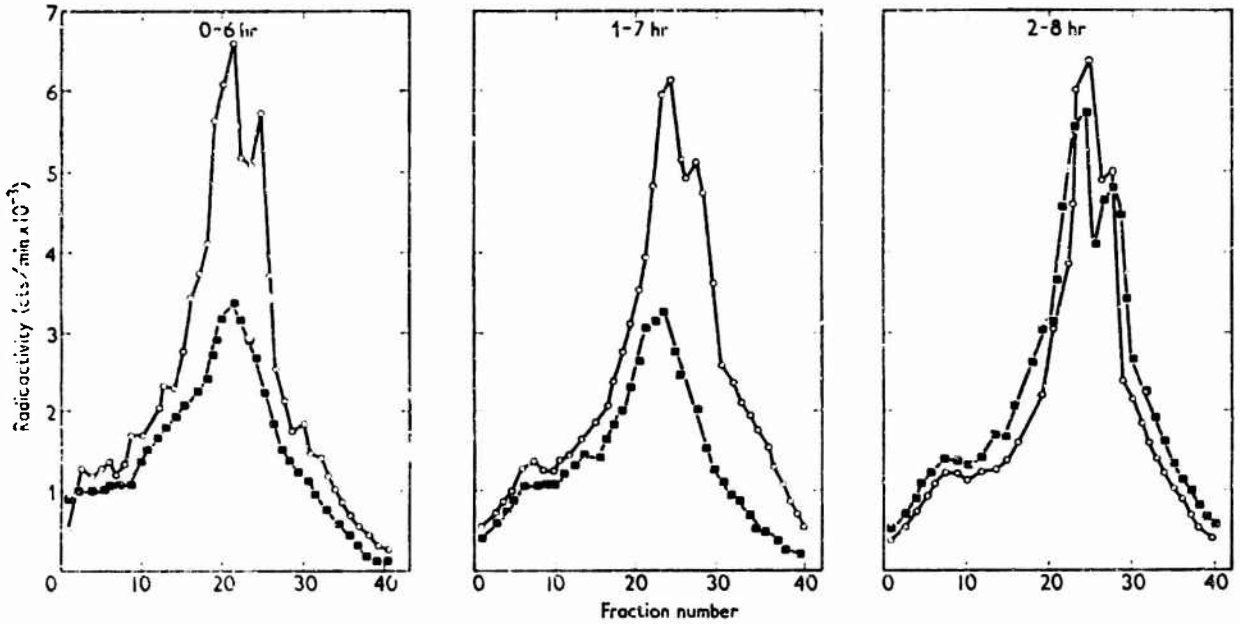

FIG. 5. The effect of time of superinfection with parental virus on the synthesis of *Ets-4* RNA. —○—○—, *Ets-4*; --■—■—, *Ets-4* and parental strain.

manifested only at or after two hours (Zebovitz & Brown, 1968). It seems reasonable to conclude, however, that encephalitis virus can influence mutant, *Ets-4*, RNA synthesis during the first hour, and that an early event following infection by the parental strain determines the level of RNA synthesis.

(h) *Effect of other viruses upon* Ets-4 *RNA synthesis*

We next determined the specificity of the requirement of the superinfecting virus for restoring control on the pattern of *Ets-4* RNA synthesis. In order to examine a number of viruses, the criterion chosen for comparing viral RNA synthesis in cells singly-infected with each virus and doubly-infected with *Ets-4* was the total RNA

TABLE 3

Inhibition of viral RNA synthesis in cells infected simultaneously with other viruses

Virus	% Inhibition in [^{14}C]uridine incorporation
Ets-4 (control)	0†
Eastern equine encephalitis	60
Vesicular equine encephalitis	60
Semliki Forest	24
Vesicular stomatitis	22
Newcastle disease	5
Sindbis	2

† [^{14}C]Uridine incorporation of *Ets-4*-infected chick embryo cells after 10 hr was approximately 15,000 cts/min/10^7 cells.

synthesis at ten hours after infection; *Ets-4* was the only virus that by itself at 37°C induced very high levels of uridine incorporation. The ten hour results are representative (Table 3) and show that Venezuelan equine encephalitis virus was as effective as our parent strain in restoring control, but vesicular stomatitis virus, Newcastle disease virus and certain other arboviruses were less effective. The reasons for this type of specificity are at present unknown, although a number of possible explanations are being investigated.

4. Discussion

The information accumulated indicates that *Ets-4* is a temperature-sensitive mutant of eastern equine encephalitis virus that may be classified as RNA$^+$ (Burge & Pfefferkorn, 1966), and that is apparently defective in viral protein synthesis. What is of greater interest, however, is that at 37°C, where the synthesis of infectious virus and viral protein synthesis are reduced compared with the parent virus, *Ets-4* induces the synthesis of excessive amounts of viral RNA (see also Yin & Lockart, 1968). The RNA products consist largely of the species that have relatively poor infectious quality (the 20 s and 27 s species) but even the normally infectious 45 s species of *Ets-4* RNA in infected cells or in purified virus had a lower specific infectivity than the parent strain, which indicates that it was biologically defective. Thus, the apparent defect in viral protein synthesis may be the result of the defect in viral RNA.

By comparing the patterns of viral RNA species produced by *Ets-4* and its parent, one could reasonably state that the control of viral RNA synthesis of *Ets-4* was defective. An attempt was then made to understand the control mechanism. As a

working hypothesis, it was assumed that a regulatory mechanism of encephalitis virus operated through a mechanism that was absent or defective in *Ets-4*-infected cells. This resulted in the increased RNA synthesis that was observed in the mutant. Our parent strain and Venezuelan equine encephalitis virus but not other related and unrelated ones could apparently restore control of RNA synthesis in doubly-infected cells to patterns closely resembling our strain. Superinfection had to occur early to be effective (one hour but not two hours after infection with *Ets-4*), suggesting the possibility that control was determined by an early event following superinfection with parent virus. "Typical" viral interference was ruled out as an explanation for the restoration of control that was observed, on the basis that none was demonstrable as judged by the titer of *Ets-4* or the parent strain in singly- or doubly-infected cells. Furthermore, it was previously shown that interference among group A arboviruses could only be demonstrated if one virus had an advantage in time or in multiplicity of infection over the (inhibited) challenge virus (Zebovitz & Brown, 1968). This type of interference was apparently not the case here. In addition, Newcastle disease virus, vesicular stomatitis virus, and two arboviruses tested that show a potential for interference if they are given an advantage (Zebovitz & Brown, 1968 and unpublished experiments), failed to restore control of *Ets-4* under conditions of simultaneous infection and higher multiplicities. One possible explanation of the presumed viral RNA regulatory mechanism is that a viral protein that is *common* to some of the serologically related group A arboviruses is the regulatory factor that is absent or defective in *Ets-4*. This is consistent with the reduced viral protein synthesis observed in *Ets-4*-infected cells. and is consistent with other known systems involving protein repressors and viruses (Eggen & Nathans, 1967; Lodish & Zinder, 1966; Ptashne, 1967; Joklik, Jungwirth, Oda & Woodson, 1967). In the case of *Ets-4* and its parent strain, one may speculate further that the viral factor may be a structural protein of the virus analogous to the results with RNA phages (reviewed by Eggen & Nathans, 1969). It has been suggested that control by the phage coat protein of the synthesis of non-coat proteins induced by the phage occurs at the transcription level (Robertson, Webster & Zinder, 1968) or at the translation level (Eggen & Nathans, 1969). We are considering somewhat analogous hypotheses that focus directly on viral RNA synthesis. However, other hypotheses are also being considered. We can summarize one of our main ideas as follows. A defect in the viral RNA in *Ets-4* results in a defect or deficiency in a viral structural protein that acts as the regulatory protein for viral RNA synthesis. Thus control is defective in the mutant.

Some of the other hypotheses that may account for the apparent loss of control of viral RNA synthesis include (1) a defective feedback inhibition or, alternatively, an end product repression mechanism (due to the defective 45 s), (2) an explanation based on the fact that the defect in viral protein synthesis permits the *Ets-4* RNA to accumulate because the latter cannot be removed from the system (the infected cell) as a mature virus and (3) the inefficient (presumed) conversion of 27 s RNA to infectious 45 s RNA by *Ets-4*. An *in vitro* system is now under development to test the hypotheses mentioned as well as to define further the parameters and mechanism for the control of viral RNA synthesis in these viruses.

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